Journal of Feline Medicine and Surgery (2004) **6**, 161–165

jfms

www.elsevier.com/locate/jfms

The use of real-time PCR in the diagnosis and monitoring of *Mycoplasma haemofelis* copy number in a naturally infected cat

J.A. Braddock[a], S. Tasker[b]*, R. Malik[a]

[a]*Faculty of Veterinary Science, The University of Sydney, New South Wales, 2006, Australia*
[b]*Department of Clinical Veterinary Science, University of Bristol, Langford House, Langford, Bristol BS40 5DU, UK*

Accepted 3 December 2003

Summary A 5-year-old male neutered cat was diagnosed with severe anaemia due to acute *Mycoplasma haemofelis* infection. Inflammatory respiratory disease was present concurrently. The cat was treated successfully using a fresh transfusion of whole blood and a 6 week course of doxycycline. The patient made a rapid recovery although the allergic airway disease subsequently required specific therapy consisting of inhaled fluticasone and salbutamol. Real-time quantitative PCR assays confirmed the presence of *M. haemofelis* DNA copies in the blood at presentation. Repeat PCR assays showed a reduction in copy number during treatment and negative PCR results were obtained both 91 and 425 days after presentation. The report describes, for the first time, the use of real-time PCR in the diagnosis and monitoring of natural *M. haemofelis* copy number, as well as the induction of long-term negative PCR status.

Introduction

Haemobartonella felis has been reclassified within the genus *Mycoplasma* as *Mycoplasma haemofelis* (Neimark et al., 2001) and '*Candidatus* Mycoplasma haemominutum' (Foley and Pedersen, 2001). These species differ in pathogenicity with *M. haemofelis* being the more pathogenic species, often causing severe haemolytic anaemia during acute infection (Berent et al., 1998; Foley et al., 1998; Westfall et al., 2001). Polymerase chain reaction (PCR) assays are known to be more sensitive than cytology for diagnosis and enable differentiation between the two species (Berent et al., 1998; Foley et al., 1998; Westfall et al., 2001). Recently a real-time PCR assay, which allows quantification of the amount of *M. haemofelis* and '*Candidatus* M. haemominutum' DNA present in blood samples, has been described (Tasker et al., 2003b). This case report documents the use of this real-time PCR assay to detect and quantify *M. haemofelis* copy number in a naturally infected Australian cat.

Case report

A 5-year-old male neutered Burmese cat, vaccinated against feline calicivirus, feline rhinotracheitis virus and panleucopenia virus, presented with a 3-day history of reduced appetite and activity. Worming and preventative flea treatment were up to date. Previous long-term history consisted of occasional coughing, attributed to low-grade bronchitis, but the frequency of coughing had increased

* Corresponding author. Tel.: +44-117928-9558; fax: +44-117928-9559
E-mail address: s.tasker@bristol.ac.uk (S. Tasker).

1098-612X/04/$ - see front matter © 2004 ESFM and AAFP. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.jfms.2003.12.004

Table 1 Selected routine haematology results

Parameter	Reference range	Units	Day 1	Day 6	Day 30
PCV	30–45	%	**10**	**26**	32
Haemoglobin	80–140	g/l	**35**	81	115
Red blood cell count	6–10	$\times 10^{12}$/l	**1.8**	**4.11**	7.8
White blood cell count	8–14	$\times 10^{9}$/l	9.7[a]	**17.7**	7.9
Eosinophils	0–0.14	$\times 10^{9}$/l	**0.58**	**1.59**	**0.79**
Platelets	300–700	$\times 10^{9}$/l	Adequate	Adequate	Adequate
Absolute aggregate reticulocyte count	>50=regenerative	$\times 10^{9}$/l	**234**	**369.9**	Not determined
Blood smear comments			Moderate anisocytosis, moderate polychromasia and many normoblasts. Epierythrocytic bodies visible	Moderate anisocytosis, moderate polychromasia and occasional normoblasts. Possible epierythrocytic bodies visible	Slight anisocytosis

PCV indicates packed cell volume. PCVs were also performed in isolation on days 2 (16%) and 4 (32%) as described in the text. Figures in bold indicate abnormal values. Haematology was performed using a Sysmex K-4500, Toa Medical Electronic Co Ltd, Kobe, Japan. PCVs, differential and reticulocyte counts were all performed manually.

[a]Count corrected for nucleated red blood cells.

in the 3 days before presentation. On clinical examination he was found to be in good body condition but was tachypnoeic (respiratory rate of 48 breaths per min) and had pale mucous membranes. He was mildly pyrexic (temperature of 39.2 °C; normal range 38–39 °C). Auscultation of the chest was difficult due to purring but bilateral wheezes were audible. His heart rate was normal (180 beats per min).

Routine haematology (Table 1) revealed a severe anaemia (PCV 10%) with regeneration (absolute reticulocyte count 234×10^{9}/l) and an eosinophilia. Many epierythrocytic bodies were present on a Diff-quik (Lab Aids, Sydney, NSW, Australia) smear along with anisocytosis, polychromasia and normoblasts. FeLV antigen and FIV antibody tests (Witness tests, Agen Biomedical Ltd, Brisbane, Australia) were negative and blood typing, using a RapidVet-H (Feline) blood typing card (DMS Laboratories, Hawksburn, Victoria, Australia), showed the cat to be type A. Real-time PCR reactions were performed in triplicate, as described previously (Tasker et al., 2003b), on DNA extracted from approximately 100 μl of blood. Quantification of copy number was achieved by reference to a standard curve generated by amplifying ten-fold dilutions of DNA from plasmid standards containing known *M. haemofelis* copy numbers. Positive and negative control reactions gave appropriate results. PCR documented the presence of 406,000 *M. haemofelis* copies per μl of blood (Table 2). Diagnostic investigation of the coughing comprised thoracic radiography which showed the presence of a marked diffuse bronchial pattern with a small area of consolidation in the right middle lung lobe.

These findings were consistent with a diagnosis of severe anaemia due to *M. haemofelis* infection, with presumptive concurrent inflammatory airway disease.

Treatment comprised intravenous administration of 50 ml of fresh type A whole blood and oral doxycycline (Vibravet, Pfizer) at 5 mg/kg/day bid for 42 days. Serial PCV determinations demonstrated a rise from 10% (day 1) to 16% (day 2) following blood transfusion, and the cat showed marked clinical improvement with resolution of tachypnoea although coughing persisted. Only occasional epierythrocytic bodies were visible in peripheral blood smears on day 2. By day 4 the PCV had risen further to 32%. On day 6 routine haematology (Table 1) showed a mild strongly regenerative anaemia with eosinophilia and basophilia. A few questionable epierythrocytic organisms remained visible on cytology. On day 30 repeat haematology revealed no significant abnormalities (Table 1) and although thoracic radiography showed resolution of the area of pulmonary consolidation, the bronchial pattern persisted. Accordingly bronchoalveolar lavage (BAL) was performed and revealed an increase in cellularity comprising predominantly neutrophils (50%) and eosinophils (some in sheet formations) (29%), with no evidence of *Aelurostrongylus abstrusus* larvae. Culture of a BAL specimen was negative. Real-time PCR was repeated on day 30 and showed less than one *M. haemofelis* copy per μl of blood (Table 2). To allow such comparison between

Table 2 Real-time PCR results

	Day 1	Day 30	Day 91	Day 425
M. haemofelis copy number	406,000	0.23	0	0

M. haemofelis copy number shown is the average of three replicate PCR reactions, performed using 2 μl of DNA extracted from approximately 100 μl of blood and eluted into a 200 μl volume. The copy number therefore represents that present per μl of blood assuming 100% efficiency of DNA extraction and amplification. Values are standardised across samples to a given amount of 28S rDNA. Treatment was started on day 1 (after sample for PCR was taken) and continued until day 42.

blood samples taken at different time points, the *M. haemofelis* copy number was standardised to a given amount of 28S rDNA, determined using the results of a 28S rDNA real-time PCR assay performed on all samples, as described previously (Tasker et al., 2003b).

Treatment for active inflammatory airway disease was started on day 30 with topical salbutamol (200 μg twice daily, Ventolin, GlaxoSmithKline) and fluticasone (250 μg twice daily, Flixotide, GlaxoSmithKline), administered with an inhaler (Padrid, 2000). The cat showed a good response to treatment and the doses of salbutamol and fluticasone were halved after 30 days of treatment, and thereafter reduced to their lowest effective doses. No abnormalities were found on clinical examination 150 days after presentation. Real-time PCR assays performed both 91 and 425 days after presentation were negative for *M. haemofelis* DNA.

Discussion

This case report documents for the first time the use of a real-time PCR assay to confirm a diagnosis of *M. haemofelis* infection and quantify the response to treatment in a naturally infected cat. Although positive PCR results for *M. haemofelis* have been reported in healthy asymptomatic cats (Tasker et al., 2003a), the visualisation of organisms on a blood smear, the high *M. haemofelis* copy number quantified by PCR (comparable to the copy number seen following experimental infection of cats (Tasker et al., 2003b)), a severe regenerative anaemia and the improvement with doxycycline all support *M. haemofelis* being the cause of clinical disease. Additionally, negative PCR results obtained 383 days after completion of a 42 day course of doxycycline (daily dose of 10 mg/kg) document effective treatment of infection.

Doxycycline (at a dose of 10 mg/kg/day for 14 days) has been shown to reduce the number of *M. haemofelis* organisms visible in blood smears from experimentally infected cats but did not reduce the number of positive results obtained using a conventional PCR assay (Dowers et al., 2002). The latter assay generated positive and negative results but gave no quantitative data on *M. haemofelis* copy number. The real-time assay used in the current case report enabled the determination of *M. haemofelis* copy number in different samples, allowing quantitative evaluation of the response to treatment. PCR was performed in triplicate to maximise sensitivity which is particularly important for samples with very low copy numbers around the sensitivity threshold of detection.

Negative PCR results obtained, in triplicate, over a year after completion of doxycycline therapy, could represent long-term clearance of *M. haemofelis*. Although the real-time PCR assay is known to be very sensitive (Tasker et al., 2003b) the possibility remains that total clearance of organism DNA did not occur in this case, but that the *M. haemofelis* copy number present in the blood was below the threshold of detection for the PCR assay. Sequestration of haemoplasma organisms in the spleen and lungs has been suggested (Simpson et al., 1978) and it may be that in such cases PCR on peripheral blood yields negative results. Evaluation of possible clearance of infection would probably require sampling of potential sites of sequestration, such as the spleen and lungs, for PCR. It is not known whether the cat was previously infected with *M. haemofelis*, as an asymptomatic carrier cat, or whether infection was acquired just prior to presentation. Routes of transmission of feline haemoplasma infection have yet to be confirmed (Tasker and Lappin, 2002) so it is not possible to speculate on how the current case acquired infection.

Although previous studies have shown tetracyclines to be effective for the treatment of anaemia in infected cats, they have not shown the consistent elimination of *M. haemofelis* infection with treatment. For example, in two experimental studies PCR tests became negative soon after starting doxycycline, but became positive again between 3 days and 5 weeks after stopping treatment (Berent et al., 1998; Foley et al., 1998). In one other study (Dowers et al., 2002), only one of four cats experimentally infected with

M. haemofelis became PCR negative during doxycycline treatment, although this cat remained PCR negative 6 months later. Direct comparison of these studies is difficult due to the use of different PCR assays, and it is unwise to draw conclusions based on a single patient. However, the previous studies all used shorter durations of doxycycline treatment (up to 3 weeks) so it may be that longer treatment periods (such as the 6 weeks used in the current case report) are required to maximise the likelihood of clearance of infection. Additionally different formulations of doxycycline are available (monohydrate, hyclate and a calcium salt) which may influence response to therapy. The current patient was treated with the monohydrate form of doxycycline. It should be noted that there have been rare reports of oesophageal stricture formation following oral administration of doxycycline tablets (McGrotty and Knottenbelt, 2002; Melendez et al., 2000). To minimise this potential complication doxycycline treatment in the current case was administered either before a meal or was followed by a small volume of water syringed per os, to encourage complete swallowing.

A blood transfusion was administered to the current case on the first day of presentation. Following transfusion on day 2 the cat's PCV was 16% (compared to 10% measured on day 1). The cat's PCV then rose dramatically to 32% on day 4. This marked increase could reflect, following effective doxycycline treatment, the release into the circulation of red blood cells previously sequestered in the spleen (due to *M. haemofelis*) infection as described previously (Maede and Murata, 1978). If the release of large numbers of sequestered red blood cells from the spleen was responsible for the rapid increase in PCV observed, it is interesting to speculate that the blood transfusion may not have been essential in the recovery of this case. The PCV decreased to 26% on day 6. Although this may reflect haemolysis due to an incompatibility in the blood transfusion, the possibility of ongoing haemolysis due to *M. haemofelis* infection cannot be ruled out.

This case shows the successful use of quantitative real-time PCR in confirming the diagnosis and monitoring infection with *M. haemofelis* in a naturally infected cat, and the induction of long-term negative PCR status. This assay is likely to be useful in the monitoring of future cases of *M. haemofelis* infection.

Acknowledgements

The real-time PCR assays were performed during a PhD studentship held by Séverine Tasker which was part-funded by the Royal College of Veterinary Surgeons Trust Fund West Scholarship for Feline Research. Richard Malik was supported by the Valentine Charlton Bequest of the Post Graduate Foundation of Veterinary Science of the University of Sydney.

References

Berent, L.M., Messick, J.B., Cooper, S.K., 1998. Detection of *Haemobartonella felis* in cats with experimentally induced acute and chronic infections, using a polymerase chain reaction assay. American Journal of Veterinary Research 59, 1215–1220.

Dowers, K.L., Olver, C., Radecki, S.V., Lappin, M.R., 2002. Use of enrofloxacin for treatment of large-form *Haemobartonella felis* in experimentally infected cats. Journal of the American Veterinary Medical Association 221, 250–253.

Foley, J.E., Harrus, S., Poland, A., Chomel, B., Pedersen, N.C., 1998. Molecular, clinical, and pathologic comparison of two distinct strains of *Haemobartonella felis* in domestic cats. American Journal of Veterinary Research 59, 1581–1588.

Foley, J.E., Pedersen, N.C., 2001. '*Candidatus* Mycoplasma haemominutum', a low-virulence epierythrocytic parasite of cats. International Journal of Systematic and Evolutionary Microbiology 51, 815–817.

Maede, Y., Murata, H., 1978. Ultrastructural observation on the removal of *Haemobartonella felis* from erythrocytes in the spleen of a cat. Japanese Journal of Veterinary Science 40, 203–205.

McGrotty, Y.L., Knottenbelt, C.M., 2002. Oesophageal stricture in a cat due to oral administration of tetracyclines. Journal of Small Animal Practice 43, 221–223.

Melendez, L.D., Twedt, D.C., Wright, M., 2000. Suspected doxycycline-induced oesophagitis with oesophageal stricture formation in three cats. Feline Practice 28, 10–12.

Neimark, H., Johansson, K.E., Rikihisa, Y., Tully, J.G., 2001. Proposal to transfer some members of the genera *Haemobartonella* and *Eperythrozoon* to the genus *Mycoplasma* with descriptions of '*Candidatus* Mycoplasma haemofelis', '*Candidatus* Mycoplasma haemomuris', '*Candidatus* Mycoplasma haemosuis' and '*Candidatus* Mycoplasma wenyonii'. International Journal of Systematic and Evolutionary Microbiology 51, 891–899.

Padrid, P., 2000. Feline asthma. Diagnosis and treatment. Veterinary Clinics of North America: Small Animal Practice 30, 1279–1293.

Simpson, C.F., Gaskin, J.M., Harvey, J.W., 1978. Ultrastructure of erythrocytes parasitized by *Haemobartonella felis*. Journal of Parasitology 64, 504–511.

Tasker, S., Binns, S.H., Day, M.J., Gruffydd-Jones, T.J., Harbour, D.A., Helps, C.R., Jensen, W.A., Olver, C.S., Lappin, M.R., 2003a. Use of a PCR assay to assess prevalence and risk factors for *Mycoplasma haemofelis* and '*Candidatus* Mycoplasma haemominutum' in cats in the United Kingdom. Veterinary Record 152, 193–198.

Tasker, S., Helps, C.R., Day, M.J., Gruffydd-Jones, T.J., Harbour, D.A., 2003b. Use of Real-Time PCR to detect and quantify *Mycoplasma haemofelis* and '*Candidatus* Mycoplasma haemominutum' DNA. Journal of Clinical Microbiology 41, 439–441.

Tasker, S., Lappin, M.R., 2002. *Haemobartonella felis*: Recent developments in diagnosis and treatment. Journal of Feline Medicine and Surgery 4, 3–11.

Westfall, D.S., Jensen, W.A., Reagan, W.J., Radecki, S.V., Lappin, M.R., 2001. Inoculation of two genotypes of *Haemobartonella felis* (California and Ohio variants) to induce infection in cats and the response to treatment with azithromycin. American Journal of Veterinary Research 62, 687–691.